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Topoisomerase IIα Is Required for Embryonic Development and Liver Regeneration in Zebrafish▽

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Topoisomerases solve the topological problems encountered by DNA throughout the lifetime of a cell. Topoisomerase IIα, which is highly conserved among eukaryotes, untangles replicated chromosomes during mitosis and is absolutely required for cell viability. A homozygous lethal mutant, *can4*, was identified in a screen to identify genes important for cell proliferation in zebrafish by utilizing an antibody against a mitosis-specific marker, phospho-histone H3. Mutant embryos have a decrease in the number of proliferating cells and display increases in DNA content and apoptosis, as well as mitotic spindle defects. Positional cloning revealed that the genetic defect underlying these phenotypes was the result of a mutation in the zebrafish *topoisomerase IIα* (*top2a*) gene. *top2a* was found to be required for decatenation but not for condensation in embryonic mitoses. In addition to being required for development, *top2a* was found to be a haploinsufficient regulator of adult liver regrowth in zebrafish. Regeneration analysis of other adult tissues, including fins, revealed no heterozygous phenotype. Our results confirm a conserved role for *TOP2A* in vertebrates as well as a dose-sensitive requirement for *top2a* in adults.

The accurate and complete replication and separation of chromosomes during mitosis is vital for the viability of cells. One potential complication encountered during cell division is the topological and tensional pressures put on DNA during these processes. Topoisomerases are responsible for enzymatically winding, unwinding, knotting, and unknotting reactions that are necessary for solving the topological problems of DNA. Genetic and biochemical studies in bacteria and yeast have revealed two classes of topoisomerases, type I and type II (38). Type I topoisomerases act on DNA by creating a temporary single-strand nick in DNA, passing the intact strand through the broken strand and then religating the nick. Type II topoisomerases, alternatively, function by binding to two double-stranded DNA molecules, generating a double-stranded break in one of the strands, passing the intact strand through the broken strand, and religating the broken strand.

During DNA replication, sister chromatids become tangled with each other as a by-product of their duplication. The primary cellular function of type II topoisomerases during mitosis is the decatenation (untangling) of sister chromatids. Consistent with this essential role, genetic studies *Saccharomyces cerevisiae* have revealed that temperature-sensitive topoisomerase II mutants (*TOP2*) are unviable at nonpermissive temperatures (10, 16, 36). These mutants arrest during M phase due to their incompletely decatenated chromatids.

Humans have two *TOP2* homologues, *topoisomerase II alpha* (*TOP2A*) and *topoisomerase II beta* (*TOP2B*). Although they are both functionally redundant with the yeast homologue, *TOP2A* and *TOP2B* are genetically unique. As the products of different loci, *TOP2A* and *TOP2B* have different expression patterns. Specifically, *TOP2A* expression peaks during M phase, whereas *TOP2B* expression remains relatively low and constant throughout the cell cycle (15, 40). TOP2A is therefore thought to be the primary topoisomerase required for the decatenation of chromosomes during mitosis. This hypothesis is supported by the fact that, similar to yeast gene deletion studies, *TOP2A* knockout mice are not viable and arrest at the four- to eight-cell stage of development (1). Although these embryos do not have any clearly tangled chromosomes, they do have teardrop-shaped nuclei, a phenotype consistent with previous reports about yeast topoisomerase mutants (36). Additionally, mitotic human cells in which *TOP2A* has been depleted quickly develop tangled chromosomes, aberrant mitoses, aneuploidy, and cell cycle arrest (5).

In addition to its role in untangling chromosomes during mitosis, TOP2A activity has also been shown to be required for RNA polymerase II (Pol II)-dependent transcription of chromatin bound DNA (22, 23). During transcription, elongation along a DNA template results in a positively supercoiled topology ahead of Pol II. In the absence of TOP2A, this accumulation of positively supercoiled DNA prevents complete transcription. Despite the fact that there are many proteins known to be part of the Pol II holo enzyme, only TOP2A is

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necessary and sufficient to overcome this topological constraint.

Due to its vital role in untangling sister chromatids, the function of TOP2A during mitosis is monitored by a decatenation checkpoint (11). Similar to the G_2/M DNA damage and spindle assembly checkpoints, the decatenation checkpoint monitors the cell's progress through the cell cycle and can affect delay or arrest (14). Compounds such as ICRF-193, which inhibits TOP2A without generating DNA damage, have been used to elucidate the mechanism of this checkpoint and dissect any differences compared to the DNA damage checkpoint (25). The decatenation checkpoint becomes activated in G_2 phase, and cells with persistent tangled chromosomes are unable to progress past metaphase (29).

In the presence of catenated chromosomes, mitotic delay is dependent upon the presence of *br*east *ca*ncer gene 1 (*BRCA1*) and the ataxia-telangiectasia and rad3 related gene (*ATR*) (8). BRCA1, likely in response to activation by ATR, accumulates during S phase at sites of decatenation. In addition to their colocalization, TOP2A is polyubiquitinated in vivo by BRCA1, an activation event that enhances the decatenating activity of TOP2A (21). When the decatenation checkpoint is activated, ATR also affects cell cycle progression by inhibiting Polo-like kinase 1 (PLK1) (9). Since PLK1 activity is required for the activation of cyclin B/cyclin-dependent kinase 1, this inhibition of PLK1 by ATR prevents mitotic progression.

Due to the vital role of TOP2A during mitosis, its expression during the cell cycle is highly controlled. Prior to G_2 phase, p21 and E2F1 bind to the cell cycle-dependent element in the *TOP2A* promoter, effectively suppressing expression (37, 41). As cells progress through G_2 phase, this suppression is lifted by a collection of activating transcription factors, including CMYB, which promote the expression of *TOP2A* (3). Additionally, the cell cycle-regulated gene UHRF1 has been shown to bind to the *TOP2A* promoter and regulate its expression in response to protein kinase A signaling (17). In zebrafish, *uhrf1* null mutants have been shown to have defects in embryonic liver growth, while adult *uhrf1* heterozygotes are incapable of normal liver regeneration after partial hepatectomy (26).

In addition to its role in normal cell cycles, TOP2A was identified early as a reliable marker for abnormally proliferating and cancerous cells (39). Unsurprisingly, topoisomerase inhibitors have well-described mechanisms and are widely used as cancer chemotherapeutics (13). Here we describe the identification and characterization of *can4*, a novel zebrafish *topoisomerase IIα* mutant. *can4* mutants display a variety of cell cycle defects that contribute to early lethality, including abnormal mitotic spindles and incompletely decatenated chromosomes during metaphase. In addition to embryonic phenotypes, we also show that adult heterozygous zebrafish have impaired liver regrowth after partial resection and that this defect is associated with decreased levels of *top2a* transcript. Finally, we demonstrate that the *top2a*-induced liver defect can be rescued with the protein kinase A stimulant forskolin.

MATERIALS AND METHODS

Cell proliferation screen. Screening procedures were done as described previously (27, 30). Adult male zebrafish of the *AB* strain were mutagenized with ethylnitrosourea and crossed to wild-type *AB* females in order to generate the F_1 heterozygous generation. F_2 haploid embryos were obtained by squeezing anesthetized F_1 females and fertilizing the eggs in vitro with UV-inactivated sperm (6). At 36 h postfertilization (hpf) haploids were collected and used for anti-phosphorylated H3 (pH3) staining.

Genetic mapping. AB strain *can4* heterozygous mutant zebrafish were outcrossed to wild-type *wik* in order to generate polymorphic mapping strains. Low-resolution mapping was done with 40 diploid mutant and 40 diploid wild-type embryos obtained from in-crossing mapping F_2 fish. Microsatellite CA markers throughout the genome were used to scan for linkage. Linkage was initially established to chromosome 12 with marker z62244. Novel primer sets that scanned bacterial artificial chromosomes (BACs) in the region were designed and utilized to perform single-stranded conformational polymorphism analysis. Novel polymorphisms were identified, including flanking markers. An in silico chromosomal walk was then performed to order the sequence of BACs between flanking markers. Once complete, the critical interval was observed to contain only two genes, including the *topoisomerase IIα* genomic sequence.

pH3 staining. Embryos were fixed in 4% paraformaldehyde overnight at 4°C and stained as described previously (27).

Mitotic spindle/chromosome staining. At 26 to 28 hpf embryos were stained for alpha- and gamma-tubulin as previously described (27).

In situ hybridization. A 2.6-kb portion of the zTOP2A open reading frame was amplified with forward and reverse primers, 5′-AGTTGCTCTCGTGTTAGTGC-3′ and 5′-CAACTACGATGACTATTTGCGAGTGG-3′, from RNA prepared from wild-type 24-hpf embryos and cloned into the pCR-Blunt II vector (Invitrogen). The resulting plasmid was digested with EcoRI and HpaI to remove 1.6 kb of the partial open reading frame, yielding a 1-kb fragment that was used to generate RNA probes. Antisense RNA probe was generated using SP6 RNA polymerase linearized with EcoRI. Sense control RNA probes were generated using T7 polymerase on KpnI-digested plasmid. Probes were purified by size exclusion chromatography. RNA in situ hybridization was performed as described previously (34).

Genotyping. *can* genotyping was performed with forward and reverse primers, 5′-CTGCAGAAACCCCTGTTAAG-3′ and 5′-AGGGGATTGACCTCTCGTTG-3′, followed by sequencing utilizing the same primers. *hi3635* mutants were genotyped as described previously (2).

Cytogenetics. Metaphase chromosomes were prepared as described elsewhere and visualized with 4′,6-diamidino-2-phenylindole (DAPI) (27).

DNA content analysis. Cell cycle analysis was performed on disaggregated 24-hpf embryos fixed in ethanol and stained with propidium iodide and analyzed by flow cytometry (20).

Apoptosis assays. At 36 hpf embryos were used to generate protein lysates using standard biochemical techniques. Caspase-3 activity in the lysates was measured with the Caspase-Glo kit (Promega), according to the manufacturer's protocol. Luminescence was measured on a luminometer. Embryos were also stained with acridine orange, as previously described (24).

Partial hepatectomy. Vivisection of anesthetized adult zebrafish was performed as described previously to remove the ventral lobe of the liver (12, 26). At least five fish of each genotype were used per experiment. Animals were allowed to recover for 3 days, at which point they were sacrificed and liver size analyzed. Forskolin-treated fish were soaked in 0.5 μM forskolin in fish water overnight, rinsed, and allowed to recover as normal.

qRT-PCR. Regenerated liver was dissected from euthanized fish and RNA was isolated utilizing Trizol (Invitrogen) reagent, according to the manufacturer's protocol. cDNA was generated utilizing the SuperScript III first-strand synthesis kit (Invitrogen) for quantitative reverse transcription-PCR (qRT-PCR). Quantitative RT-PCR was performed utilizing the Sybr GreenER reagent system (Invitrogen). Primers against the *top2a* and the control, β-*actin*, were used as described previously (26, 32).

Morpholinos. An ATG morpholino against the *top2a* transcript with the following sequence was obtained from Gene Tools: TCAGAGGTCCTTCTGTTCCAGCCAT. Nuclei were visualized with DAPI.

RESULTS

***can4* is a zebrafish cell cycle mutant.** In order to identify genes important for embryonic cell division, we performed an F_2 ethylnitrosourea mutagenesis screen in zebrafish for homozygous recessive mutations. Mutagenized F_0 male zebrafish were outcrossed in order to generate heterozygous F_1 adults. Haploid F_2 offspring were probed at 36 hpf with an antibody against the mitotic marker phosphorylated serine-10 of histone H3 (pH3). One of the mutants recovered, *can4*, displayed a

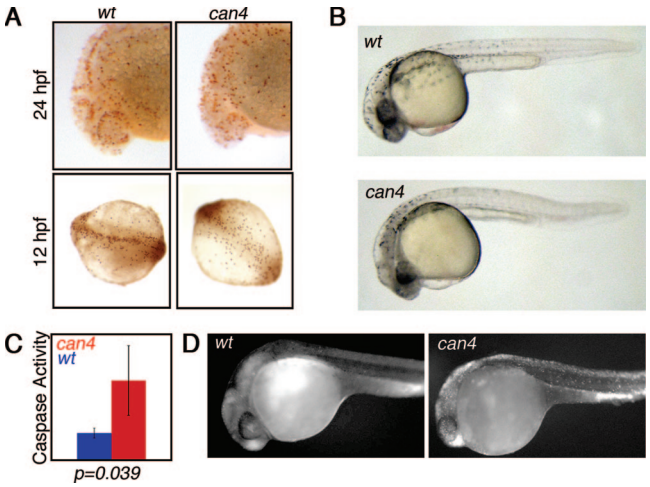

FIG. 1. *can4* embryos have decreased numbers of mitotic cells, increased apoptosis, increases in ploidy, and multipolar spindles. (A) pH3 staining in zebrafish embryos identifies mitotic cells in wild-type siblings and *can4* homozygous mutants at 12 and 24 hpf. (B) *can4* mutants have ventral tail curvature and brain necrosis compared to wild type. (C and D) Assays for caspase-3 activity in embryo protein extracts (C) and acridine orange staining (D) indicate increased apoptosis in *can4* mutants.

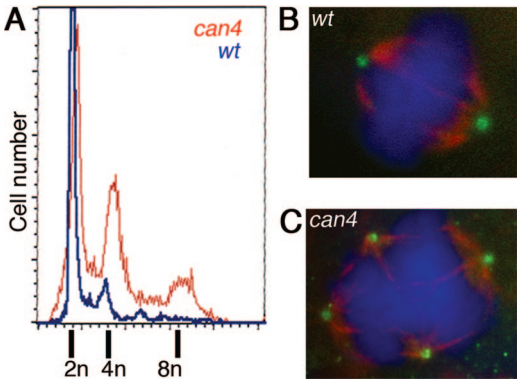

FIG. 2. *can4* mutant embryos have increased DNA content and mitotic spindle defects. (A) DNA content analysis of *can4* (red) and wild-type (blue) embryos as measured by flow cytometry. *can4* mutants have significant increases in the number of aneuploid and polyploid cells. (B and C) Mitotic spindle staining of normal and *can4* 24-hpf embryos. Spindles were stained with α-tubulin (red) and γ-tubulin (green). DNA was counterstained with DAPI (blue). Of observed spindles in *can4* mutants, 24% were multipolar ($P = 0.0002$).

slight decrease in pH3 staining compared to wild-type siblings (Fig. 1A). This phenotype was observable as early 12 hpf. Quantification of pH3-positive cells from 12-hpf embryos revealed that wild-type embryos contain 192 (±9) cells while *can4* mutant embryos contain only 161 (±12) cells per embryo ($P = 0.038$). Additionally, homozygous mutant embryos have high levels of brain necrosis and develop abnormal tail curvature at 24 hpf (Fig. 1B). These phenotypes persist until the embryos die at 4 to 5 dpf.

To determine if this decrease in proliferation was accompanied by a corresponding increase in cell death, we assayed the relative levels of apoptosis in our fish. Protein extracts from *can4* mutant and wild-type embryos were harvested and tested for activated caspase activity. In this assay, *can4* mutant extracts showed a sevenfold increase in caspase-3 activity compared to their wild-type siblings (Fig. 1C) ($P = 0.039$). Acridine orange staining of 24-hpf embryos revealed that the majority of this apoptosis was occurring in the brain and along the neural tube (Fig. 1D).

DNA content analysis revealed that *can4* mutants have increased polyploidy and aneuploidy (Fig. 2A). This defect indicates a required role of the *can4* gene in maintaining genome stability. To further probe the cause of aneuploidy and polyploidy seen by fluorescence-activated cell sorting, we visualized mitotic spindles in 24-hpf mutant embryos by costaining for alpha-tubulin and gamma-tubulin (Fig. 2B and C). In a wild-type tail, spindles were bipolar and normally formed ($n = 50$). By contrast, 23% (11/48) of spindles observed in *can4* mutants were multipolar ($P = 0.0002$). Taken together, these results suggest that *can4* mutants have a problem accurately segregating their chromosomes as well as regulating mitotic spindle formation.

***Topoisomerase IIα* is the *can4* gene.** We utilized positional cloning to discover the genetic defect responsible for the observed embryonic phenotypes. The *can4* mutation was mapped to a 1.5-cM region of chromosome 12 (Fig. 3A). The assembly of BACs across the region and analysis of this critical interval led to the identification of the candidate gene, *top2a*. Sequence analysis of mutant cDNA revealed a polymorphism that was unique when compared to the wild-type allele at amino acid 683 of the predicted protein sequence. The polymorphism consists of 11 additional nucleotides in the cDNA which causes a frameshift and premature translational stop. Sequencing the genomic DNA revealed that the *can4* polymorphism is an intronic mutation that creates a cryptic splice donor in intron 14, which allows for the inclusion of 11 intronic nucleotides in the *can4* cDNA (Fig. 3B and C). The *can4* mutation occurs nearly halfway through the expected polypeptide, well before the catalytic Tyr-839 (Fig. 3D).

top2a was verified as the gene responsible for the *can4* phenotypes by crossing *can4* heterozygotes to the previously identified *top2a* mutant *hi6535* (2). The *hi3635* allele was identified as part of an insertion-based mutagenesis screen for genes required for embryonic survival. Since the viral insertion in *hi3635* disrupts the first exon of *top2a*, the allele is thought to be a null (Fig. 3D). As expected, the *hi3635* allele failed to complement the *can4* mutation and resulted in a clutch of embryos with the predicted Mendelian ratio of 92/404 (24%) of the progeny exhibiting morphological phenotypes similar to both *can4* and *hi3635*.

In order to determine the endogenous expression pattern of *top2a* during development, RNA in situ hybridization was performed (Fig. 4). A fragment, 5′ in relation to the *can4* mutation of the zebrafish *top2a* open reading frame was cloned and used for in situ hybridization (Fig. 3D). At 36 hpf, wild-type embryos express *top2a* throughout the head and hindbrain proliferative zone (Fig. 4A). Conversely, homozygous *can4* mutant embryos have very little signal, suggesting nonsense-mediated decay of abnormal *can4* transcripts and extinction of any maternally deposited *top2a* mRNA (Fig. 4B). In wild-type embryos, *top2a* is expressed early and ubiquitously throughout early embryogenesis (Fig. 4D to F). The presence of *top2a*

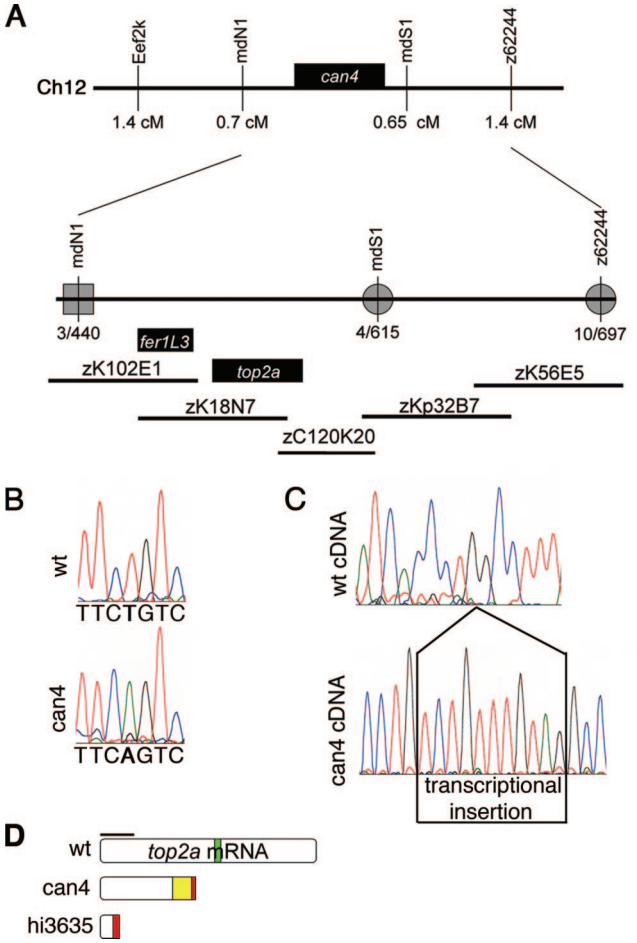

FIG. 3. *Topoisomerase IIa* is the *can4* gene. (A) Positional cloning of the *top2a* gene. Depicted is the *top2a* locus on zebrafish chromosome 12 with the positions of key markers. Below is a reconstruction of the BAC assembly for the region that includes the locations of the only two genes in the critical interval, *TOP2A* and *fer1L3*. (B) Analysis of the genomic sequence identified a T-to-A point mutation in intron 14. (C) Sequence analyses of *top2a* cDNA isolated from 24-hpf wild-type (top) and *can4* mutant (middle) embryos. The *can4* transcript includes 11 nucleotides that result in a translational frameshift and early stop. (D) Schematic of wild-type and mutant *top2a* transcripts. Stop codons are highlighted in red. The frameshift observed in *can4* transcripts is highlighted in yellow. The location of the catalytic residue is indicated in green. The location of the probe used for in situ hybridization is also shown.

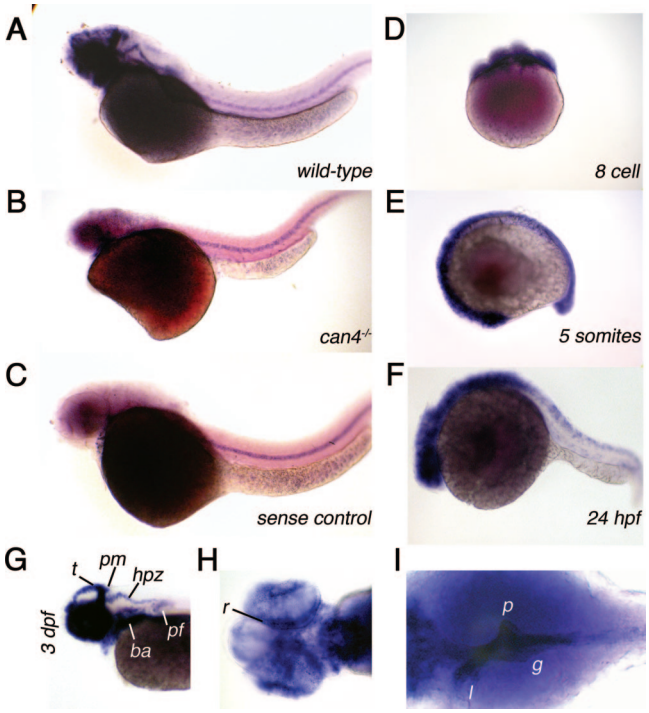

FIG. 4. *Topoisomerase IIa* expression in the zebrafish embryo. (A and B) The 36-hpf wild-type and *can4* embryos were stained for a probe against the *top2a* transcript. Robust head staining at this age distinguishes embryos of each genotype. (C) Sense control probe, showing no specific staining above background. (D and E) Expression of *top2a* in eight-cell and five-somite wild-type embryos shows diffuse, ubiquitous staining throughout early embryogenesis. (F) At 24 hpf, *top2a* expression is observed diffusely in the head as well as the neural tube. (G and H) Lateral and dorsal views of 3-dpf embryos indicate that *top2a* expression is restricted to the tectum (t), posterior midbrain (pm), hindbrain proliferative zone (hpz), branchial arches (ba), pectoral fin buds (pf), and retina (r). (I) Also seen from the dorsal view, *top2a* expression is also seen in the liver (l), pancreas (p), and gut (g) at 3 dpf.

transcript early in embryogenesis underscores the importance of the cell cycle genes like *top2a* to early cell division and strongly suggests that these early divisions are dependent on maternally derived transcripts. At 24 hpf, *top2a* expression becomes restricted to proliferating tissues, and by 3 dpf neural expression is limited to the tectum, posterior midbrain, and hindbrain proliferative zone (Fig. 4G) (33). Expression in 3-dpf embryos was also observed in the branchial arches, retina, fin buds, liver, pancreas, and gut (Fig. 4G to I).

Topoisomerase IIα is required for chromosome decatenation during mitosis. TOP2A has been shown to be absolutely required for mammalian development as well as for human somatic cell cycles (1, 2, 5). Although other topoisomerases are capable of condensing replicated chromatin, TOP2A alone is required for decatenating mitotic chromosomes. In order to assess whether *top2a* is required for chromosome decatenation in zebrafish, metaphase chromosome spreads were analyzed (Fig. 5A to C). Consistent with other systems, *top2a* deficiency in zebrafish results in incompletely decatenated metaphase chromosomes. Figure 5A shows the normal spectrum of condensed chromosomes. Tangled chromosomes are characterized by poor chromosome spreading and poor resolution of individual chromosomes (Fig. 5B and C). Unresolved linkages between sister chromosome telomeres were observed, as were aneuploid and polyploid cells. Interestingly, chromosomes from both *hi3635* (not shown) and *can4* mutant embryos had no observable defect in chromosome condensation. These results suggest that Top2a is necessary for chromosome decatenation but does not have a required role in chromosome condensation in zebrafish.

In order to further probe the role of maternally derived transcripts, an ATG morpholino targeted against the *top2a* mRNA was injected into the yolk of one-cell embryos. Consistent with high levels of maternally deposited *top2a* protein and transcript, morpholino concentrations at and below 100

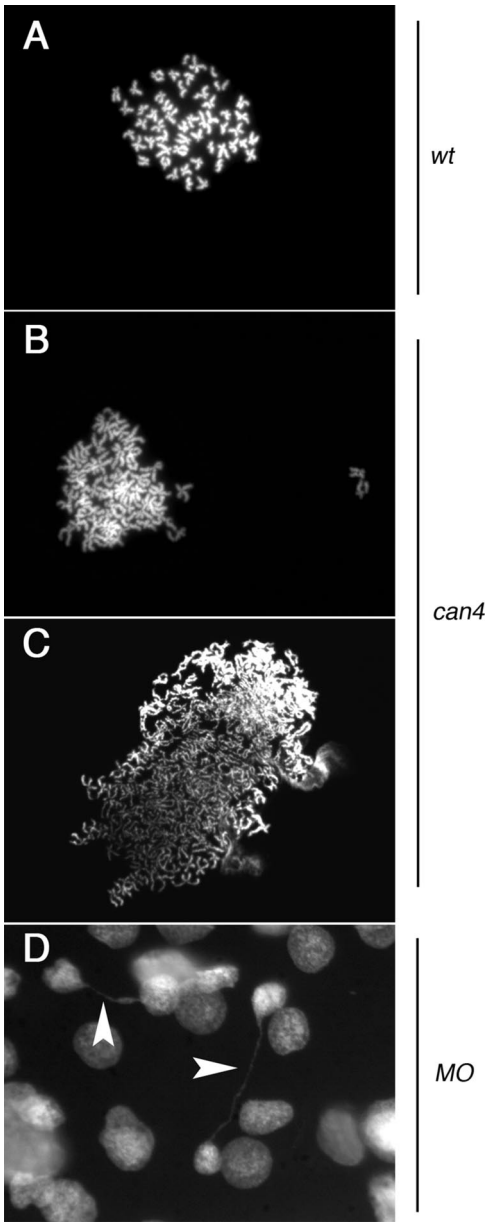

FIG. 5. Zebrafish embryos deficient in *Topoisomerase IIa* fail to decatenate their chromosomes during mitosis. Shown are representative metaphase spreads from wild-type (A) and *can4* mutant (B and C) embryos. Wild-type chromosomes are condensed, discrete, and spread evenly. Although *can4* mutant metaphases display condensed chromosomes, they are clearly tangled and do not spread normally. Polyploidy and aneuploidy were easily observed. (D) A *top2a* ATG morphant was found to exhibit bridged nuclei.

μM had no effect on injected embryos. At doses in excess of 1 mM, general toxicity as early as the 64-cell stage was visible. However, the absence of tangled chromosomes suggests this phenotype was nonspecific and the result of morpholino-induced toxicity. Embryos injected with morpholino at the intermediate concentration of 500 μM developed normally until 24 hpf, at which point they developed brain necrosis and tail curvature similar to the phenotypes of *can4* homozygous mutant animals. Although catenated metaphase chromosomes were not observed in these animals, a small number of bridged nuclei were found in these animals. This phenotype was previously described in the *top2a* knockout mouse and is thought to be the result of cytokinesis in the presence of incompletely segregated chromosomes (Fig. 5D) (1). Arguing that the effect of this *top2a* morpholino is specific, animals injected with similar concentrations of a p53 morpholino control were not observed to contain bridged nuclei.

***Topoisomerase IIα* is haploinsufficient for liver regeneration.** Due to its required and transcriptionally regulated role in cell division, *TOP2A* has long been used as a marker of proliferation for both normal and cancerous tissues. Uhrf1 is a known positive regulator of *TOP2A* activity. Recent studies in zebrafish have implicated Uhrf1 as an important regulator of liver growth and regeneration (26). Arguing for a tissue-specific role, heterozygous *uhrf1* mutant fish are unable to regenerate their livers after partial hepatectomy but have normal fin regeneration. However, even though these studies show a concurrent decrease in *top2a* transcript in *uhrf1* heterozygotes, it is not possible to determine if this observation is due to a direct effect of Uhrf1 on *top2a* transcription or if decreased *top2a* levels are a secondary effect merely reflecting the decreased mitotic index of mutant liver regrowth. We hypothesized that Top2a activity in adult liver regulates liver regrowth after resection. To test this hypothesis we analyzed the effect of *top2a* heterozygosity on liver regrowth by performing partial hepatectomies on *can4* and *hi3635* heterozygous fish. Shown are representative animals 3 days after resection for both wild-type sibling and $top2a^{+/-}$ fish (Fig. 6A and B). Liver regrowth was quantified and found to be significantly decreased in both *can4* and *hi3635* heterozygotes following partial resection (Fig. 6C).

In order to determine if *top2a* heterozygosity causes organism-wide regenerative defects, we assessed fin regrowth in a partial amputation assay. In agreement with the liver-specific sensitivity to *uhrf1* heterozygosity, *top2a* heterozygosity had no observable effect on fin regeneration at 10 days postamputation (Fig. 6E). One possible explanation for this tissue specificity is reduced basal transcription of the *top2a* locus in *hi3635* heterozygous liver. In order to test this possibility, we analyzed homeostatic liver and fin from *hi3635* heterozygous and sibling fish for baseline levels of *top2a* transcript. qRT-PCR analysis revealed no difference in *top2a* transcript levels between heterozygous mutants and siblings in either tissue (Fig. 6F). In order to confirm that the defect in liver regeneration was associated with the decreased level of *top2a* transcript in *hi3635* heterozygous liver compared to wild-type siblings, we performed qRT-PCR on regenerating liver 1 day postresection. Unlike homeostatic liver, our analysis of 10 *hi3636* heterozygotes and 10 sibling wild types revealed that *top2a* transcripts were depressed in regenerating liver from *hi3635* heterozygotes compared to their wild-type siblings ($P = 0.0035$) (Fig. 6F).

Protein kinase A directly phosphorylates UHRF1 in response to forskolin-induced cyclic AMP (cAMP) signaling (35). In order to determine if this activation is sufficient to overcome the regeneration defect in our *top2a* heterozygotes, we challenged *hi3635* heterozygotes with the cAMP-activating drug forskolin. After liver resection, fish were either treated with 0.5 μM forskolin overnight or with 1% dimethyl sulfoxide as a control. While forskolin had a minimal effect on liver

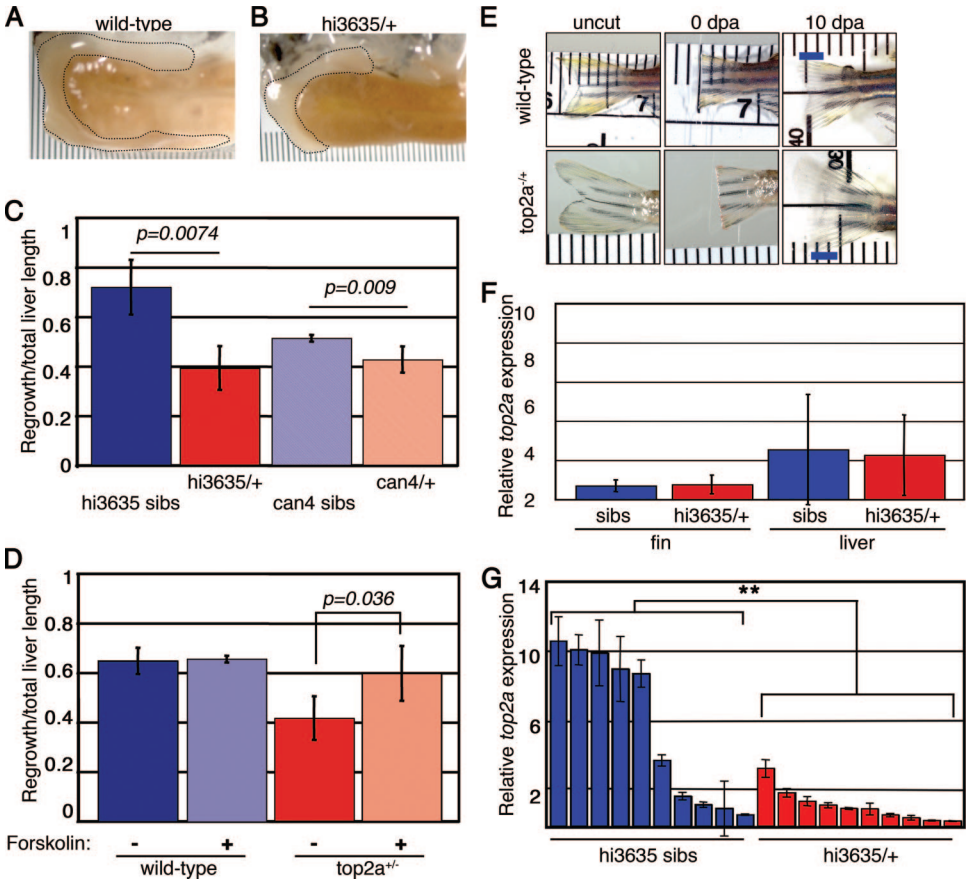

FIG. 6. *top2a* is haploinsufficient for liver regrowth in zebrafish. (A and B) Sibling wild-type and *hi3635* heterozygous fish are shown 3 days postresection. The ventral and left lateral lobes of the liver are outlined. (C) Liver regrowth in *can4* and *hi3635* heterozygotes was compared to age-matched siblings and was observed to be significantly different, with *P* values of 0.0074 and 0.009, respectively. An unpaired *t* test was used for these calculations. (D) Forskolin is able to rescue the liver regeneration defect in *hi3635* heterozygotes ($P = 0.036$). (E) Sibling wild-type or *can4* heterozygous adult fin was amputated and allowed to regrow for 10 days. Representative fish are shown prior to amputation, immediately after, and 10 days postamputation (dpa). The length of regrowth is indicated. (F) No difference was found in relative expression of *top2a* mRNA found in liver or caudal fin of *hi3635* heterozygous (red) or wild-type sibling fish (blue). (G) Relative expression of *top2a* mRNA from regenerating liver was analyzed 1 day postresection. Ten animals of each genotype, *hi3635* heterozygous (red) and sibling wild types (blue), were used for this experiment. A paired, two-tailed Student's *t* test was used to compare the relative expression of *top2a* between the groups. **, the difference was found to be significant ($P = 0.0035$).

regrowth in sibling wild-type fish, the regrowth in *hi3635* heterozygotes was restored to normal levels (Fig. 6D). These data indicate that functional defects in liver growth caused by *top2a* haploinsufficiency can be rescued by activating cAMP. Additionally, these results support the hypothesis that Uhrf1 acts by promoting *top2a* transcription during liver regeneration in zebrafish.

DISCUSSION

In the present study we describe the identification of *can4*, a novel zebrafish *topoisomerase IIα* mutant. *can4* was identified in a forward genetic screen for genes required for vertebrate cell cycles. The ability to identify cell cycle mutants in zebrafish epitomizes the unique utility of the system in studying cell cycle genetics. The requirement of *top2a* during zebrafish development is consistent with the well-known role of TOP2A in chromosome decatenation. Additionally, results in zebrafish embryos lacking functional *top2a* alleles are in agreement with the observed requirement of *TOP2A* in mouse development, yeast division/fission, and cell cycle progression in human cells. However, our observations of catenated chromosomes, aneuploidy, and polyploidy in *can4* mutants indicate a defective decatenation checkpoint during early zebrafish development. The deficiencies in decatenation checkpoint signaling in zebrafish appear similar to the observed defects in embryonic stem (ES) cells. Indeed, ES cells cultured in vitro are poorly able to sense catenated chromosomes and become better able to sense chromosome entanglement as they are forced to differentiate. Like ES cells, zebrafish embryos lacking Top2a activity accumulate aberrant mitoses and aneuploidy instead of succumbing to checkpoint arrest.

This similarity between zebrafish embryos and ES cells may reflect the inherent nature of developmental cell cycles. The deemphasis of G_2 checkpoints during development, including the decatenation checkpoint, may simply reflect the rapid nature of development. Because early zebrafish embryos have a yolk that contains the protein and mRNA complement re-

quired for rapid development, early cell divisions are more similar to the cyclic progression of S and M phases without the benefit of normal checkpoints. The fact that mouse Top2a-deficient embryos, but not ES cells, arrest almost immediately suggests that mammalian systems may have developed alternative mechanisms for sensing decatenation deficiencies in vivo. The abundance of *top2a* transcript in early embryos further underscores the importance of this transcript and implicates maternal transcript as a primary source of *top2a* mRNA and protein in early zebrafish embryos.

The accumulation of multipolar spindles as the primary mitotic defect in our *can4* embryos is consistent with the role of TOP2A in the resolution of sister centromeres during mitosis. Sumoylation of TOP2A protein during the metaphase-anaphase transition by RanBP2, a SUMO E3 ligase, is required for proper centromeric localization (7). The lack of RanBP2 results in the mislocalization of several centromeric proteins that are required for normal kinetochore-microtubule interactions. Similar to *top2a*-deficient zebrafish embryos, the primary spindle defect in these cells is multipolar spindles (19). Although it is unknown whether the polyploidy is the primary defect or a residual consequence from an earlier aberrant mitotic event, it seems clear that the localization of TOP2A to the centromeres is required for both complete chromosome decatenation as well as proper kinetochore-microtubule dynamics.

It is interesting that adult regenerating liver is particularly sensitive to *top2a* heterozygosity. This observation is consistent with the hypothesis that liver is particularly sensitive to certain genes, like *UHRF1*, that directly regulate *TOP2A* transcription. It is important to note that because we were unable to verify the absence of catenated chromosomes in liver cells of forskolin-treated animals, it is possible that forskolin may be causing DNA damage in *top2a*$^{+/-}$ liver by forcing aberrant cell cycle progression.

The required role of Top2a in zebrafish liver regeneration is particularly intriguing in the context of TOP2A as a chemotherapeutic target. Some gastrointestinal cancers, including colorectal carcinoma, are resistant to topoisomerase poisons (28). Molecularly, these tumors are characterized by gross overactivation of Wnt signaling, resulting in the accumulation of β-catenin (31). Surprisingly, β-catenin has recently been found to enhance the decatenating activity of TOP2A in vitro (18). Recent work from our lab has revealed a strong sensitivity of liver, during development as well as adult regeneration, to Wnt signaling in zebrafish and mouse (12). In the context of cancer, it is possible that the gross overexpression of β-catenin inhibits the efficacy of topoisomerase poisons. Our data also support this hypothesis by indicating that nontransformed liver cells are sensitive to *top2a* levels.

The identification of *can4* confirms the evolutionarily conserved role of topoisomerase IIα in eukaryotic mitosis. Future studies into the interplay of self-renewal genes with cell cycle machinery, such as TOP2A, should deepen the understanding of vertebrate regenerative biology. Additionally, *top2a*-deficient zebrafish embryos may provide a useful platform for studying chemoresistance. Recent studies indicate that Top2a expression is inversely correlated with response to chemotherapies (4). *top2a* heterozygous zebrafish embryos may serve as a useful platform for dissecting the genetic pathways of chemosensitivity.

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